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ZEB1-Mediated Transcriptional Upregulation of circWWC3 Promotes Breast Cancer Progression through Activating Ras Signaling Pathway

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Zinc finger E-box binding homeobox 1 (ZEB1) has been widely recognized as an important driver of tumor growth and metastasis. However, nothing is known about ZEB1-regulated circular (circ)RNAs in cancer. In the current study, we evaluated the function of a novel ZEB1-regulated circRNA derived from the WWC3 gene locus, circWWC3 in breast cancer progression. We found that ZEB1 upregulated circWWC3 expression but not the linear WWC3 mRNA expression. circWWC3 is highly expressed in breast cancer tissues and is associated with the poor prognosis of breast cancer patients. Silencing of circWWC3 significantly suppresses the proliferation, migration, and invasion of breast cancer cells. Mechanically, circWWC3 upregulates multiple oncogenes' expression of the Ras signaling pathway through acting as the sponge of microRNA (miR)-26b-3p and miR-660-3p. Moreover, short hairpin (sh)RNA-mediated knockdown of circWWC3 partially antagonized ZEB1-mediated breast cancer growth and metastasis *in vivo*. Our findings reveal that ZEB1-mediated upregulation of circWWC3 promotes breast cancer progression through activating Ras signaling pathway, which provides a potential therapeutic target and prognostic biomarker for breast cancer.

INTRODUCTION

Zinc finger E-box binding homeobox 1 (ZEB1), which is a transcription factor, has been widely considered to regulate a broad range of biological functions in breast cancer.[1–7] However, the detailed mechanisms how ZEB1 regulates breast cancer progression remain to be further elucidated.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs that are derived from precursor (pre)-mRNA back splicing and are covalently closed transcripts and therefore, are highly stable compared to their linear types.[8–10] Accumulating evidence demonstrated that circRNAs are involved in the development of various carcinomas through acting as microRNA (miRNA) sponges, forming RNA-protein or RNA-RNA complexes and regulating targeted gene splicing and transcription.[11–15] It has also been demonstrated that circRNAs are generated cotranscriptionally and that canonical pre-mRNA splicing can compete with circularization of exons.[16] For instance, estrogen receptor β (ERβ) has been reported to transcriptionally suppress circATP2B1 expression, leading to reduced microRNA (miR)-204-3p, which increased fibronectin 1 (FN1) expression and enhanced clear cell renal cell carcinoma (ccRCC) cell invasion.[17] Transcription factor androgen receptor (AR) was reported to suppress circHIAT1 expression, which promoted ccRCC development.[18] Twist1 binds the Cul2 promoter to activate its transcription and selectively promote expression of Cul2 circRNA but not mRNA, which regulated vimentin expression and promoted hepatocellular carcinoma progression.[19] As a transcription factor, how ZEB1 regulates circRNA expression and functions in breast cancer progression are still poorly understood.

In the present study, we found ZEB1 promotes the transcription of pre-WWC3 through binding to the WWC3 promoter and upregulates circWWC3 expression but not WWC3 mRNA expression. circWWC3 was highly expressed in breast cancer tissues and indicated a poor prognosis of breast cancer patients. Functionally, the circular form but not the linear form of WWC3, enhanced the proliferation, migration, and invasion of breast cancer cells. Mechanically, circWWC3 upregulated multiple oncogenes' expression of the Ras signaling pathway by absorbing miR-26b-3p and miR-660-3p. A

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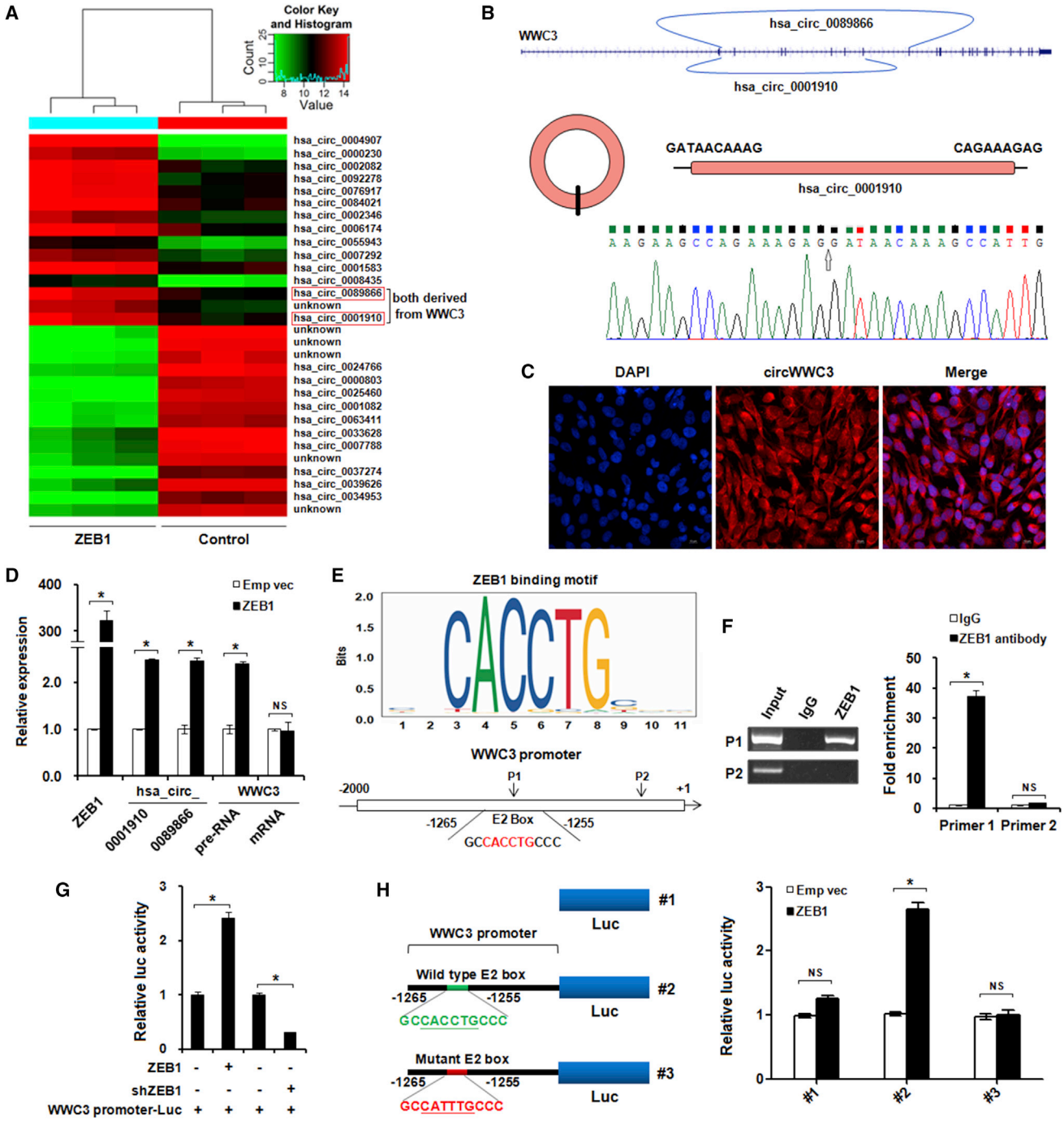
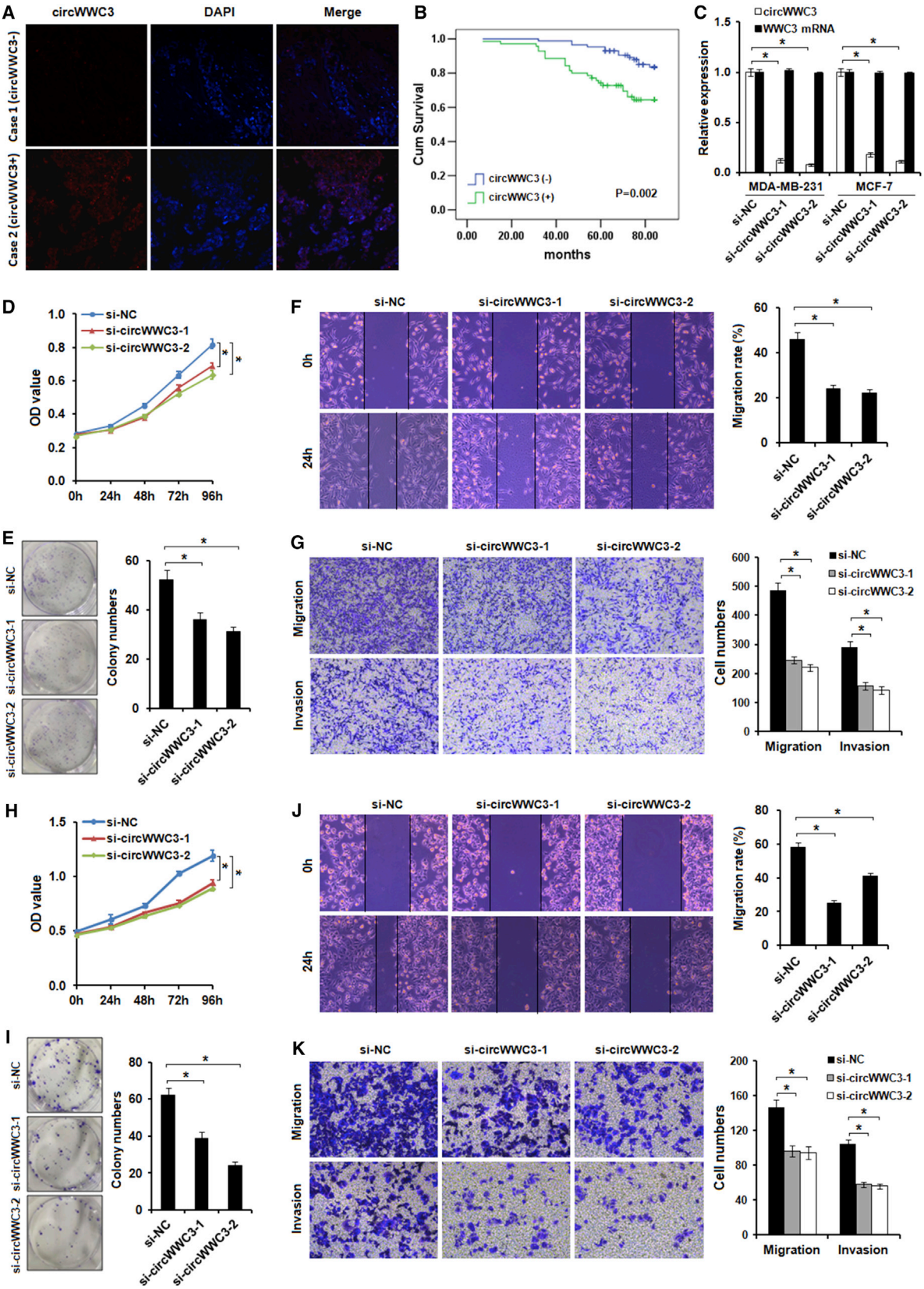


Figure 1. ZEB1 Directly Upregulates Circular WWC3 (circWWC3) Expression

(A) Heatmap of hierarchical clustering indicates the top 15 upregulated (red) and downregulated (green) circRNAs. Two circRNAs (hsa_circ_0089866 and hsa_circ_0001910), which are derived from the same gene WWC3, were selected for further study. (B) The genomic structure of hsa_circ_0089866 and hsa_circ_0001910. The expression of hsa_circ_0001910 in MDA-MB-231 cells was detected by RT-PCR and then validated by Sanger sequencing. (C) Fluorescence *in situ* hybridization (FISH) demonstrated that circWWC3 preferentially localized in cytoplasm. (D) The expression of ZEB1, WWC3 circRNAs, mRNA, and pre-mRNA in ZEB1-overexpressed MDA-MB-231 cells was detected by qRT-PCR. *$p < 0.05$; not significant (NS), $p > 0.05$. (E) The putative ZEB1 binding motif in the WWC3 promoter was analyzed by JASPAR online database. (F) ChIP-PCR, using the specific antibody against ZEB1, was performed to validate whether ZEB1 could bind to the WWC3 promoter. *$p < 0.05$; NS, $p > 0.05$. (G) Dual-luciferase reporter assay was used to detect the effect of ZEB1 on the activity of the WWC3 promoter. *$p < 0.05$. (H) The luciferase reporter assay was performed in MDA-MB-231 cells cotransfected with wild-type or mutant WWC3 promoter luciferase constructs and ZEB1 overexpression vector. *$p < 0.05$; NS, $p > 0.05$.



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breast cancer *in vivo* study revealed that short hairpin (sh)RNA-mediated knockdown of circWWC3 partially antagonized ZEB1-mediated breast cancer growth and metastasis. Our study revealed a novel function of the ZEB1/circWWC3 axis through activating the Ras signaling pathway in breast cancer progression and provided novel insights into underlying mechanism of breast cancer.

RESULTS

ZEB1 Directly Upregulates circWWC3 Expression

To investigate how circRNAs are regulated by ZEB1, we analyzed the circRNA expression profile after ZEB1 transfection using the Arraystar human circRNA microarray in MDA-MB-231 cells. The circRNA expression profile was put into Tables S2 and S3. Based on the microarray results, we filtered out 15 upregulated and 15 downregulated circRNAs (Figure 1A). We noted that two circRNAs (hsa_circ_0089866 and hsa_circ_0001910), which are derived from the same gene *WWC3*, were upregulated after ZEB1 transfection (Figure 1A). The genomic structure shows hsa_circ_0089866 contains eight exons from exon 2 to exon 9 (1,068 nt), and hsa_circ_0001910 contains seven exons from exon 2 to exon 8 (825 nt) from the *WWC3* gene flanked by long introns on either side (Figure 1B; Figures S1A and S1B). The distinct products of the expected sizes were amplified using outward-facing primers in breast cancer tissues and cell lines (Figures S1C and S1D). These two circular isoforms of WWC3 were resistant to RNase R, whereas the linear form RNA was significantly reduced after RNase R treatment (Figure S1E). Actinomycin D treatment revealed that the circRNA isoforms were highly stable, with the transcript half-life exceeding 24 h, whereas the associated linear transcript exhibited a half-life of <4 h (Figure S1F). The qRT-PCR results using outward-facing primers showed that hsa_circ_0001910 has a higher abundance than hsa_circ_0089866 in breast cancer cells and tissues (Figures S2A and S2B). Sequencing data in ~1,000 human cancer cell lines also showed that hsa_circ_0001910 is highly expressed in breast cancer (BRCA) lineage (Figure S2C).[20]

Therefore, we focused on hsa_circ_0001910 (termed circWWC3) in our present study, and the PCR product of circWWC3 was confirmed by Sanger sequencing (Figure 1B). Fluorescence *in situ* hybridization (FISH) analysis demonstrated the circular form of WWC3, preferentially localized in the cytoplasm (Figure 1C). To further confirm the results of the circRNA microarray, we examined the expression of WWC3 circRNAs, mRNA, and pre-mRNA in ZEB1-overexpressed MDA-MB-231 cells. Our results showed that enforced expression of ZEB1 increased the expression of circWWC3, including hsa_circ_0089866 and hsa_circ_0001910. Interestingly, ZEB1 overexpression increased the WWC3 pre-mRNA level, but did not affect the expression of WWC3 mRNA (Figure 1D). To explore the transcription regulation of ZEB1 on circWWC3, we analyzed the promoter sequence of WWC3 and found a binding motif of ZEB1 on −1,265 bp to −1,255 bp upstream of the transcription start site (Figure 1E). Chromatin immunoprecipitation (ChIP)-PCR analysis revealed the occupancy of ZEB1 on −1,265 bp to −1,255 bp of the WWC3 promoter, suggesting ZEB1 directly binds to the WWC3 promoter (Figure 1F). The dual-luciferase reporter assay showed ZEB1 overexpression increased the WWC3 promoter activity, whereas ZEB1 knockdown decreased WWC3 promoter activity (Figure 1G). To further demonstrate whether WWC3 is a direct transcriptional target gene of ZEB1, the luciferase reporter assay was performed in MDA-MB-231 cells cotransfected with wild-type or mutant WWC3 promoter luciferase construct and ZEB1 overexpression vector. The results indicated that the 2-kb promoter region (−1,265 bp to −1,255 bp upstream of the WWC3 transcription start site) is essential for ZEB1-mediated transcriptional activation of WWC3 gene (Figure 1H). Taken together, our results suggested as a transcription factor, ZEB1 increases circWWC3 expression through directly binding to WWC3 promoter.

circWWC3 Expression Is Associated with Breast Cancer Progression

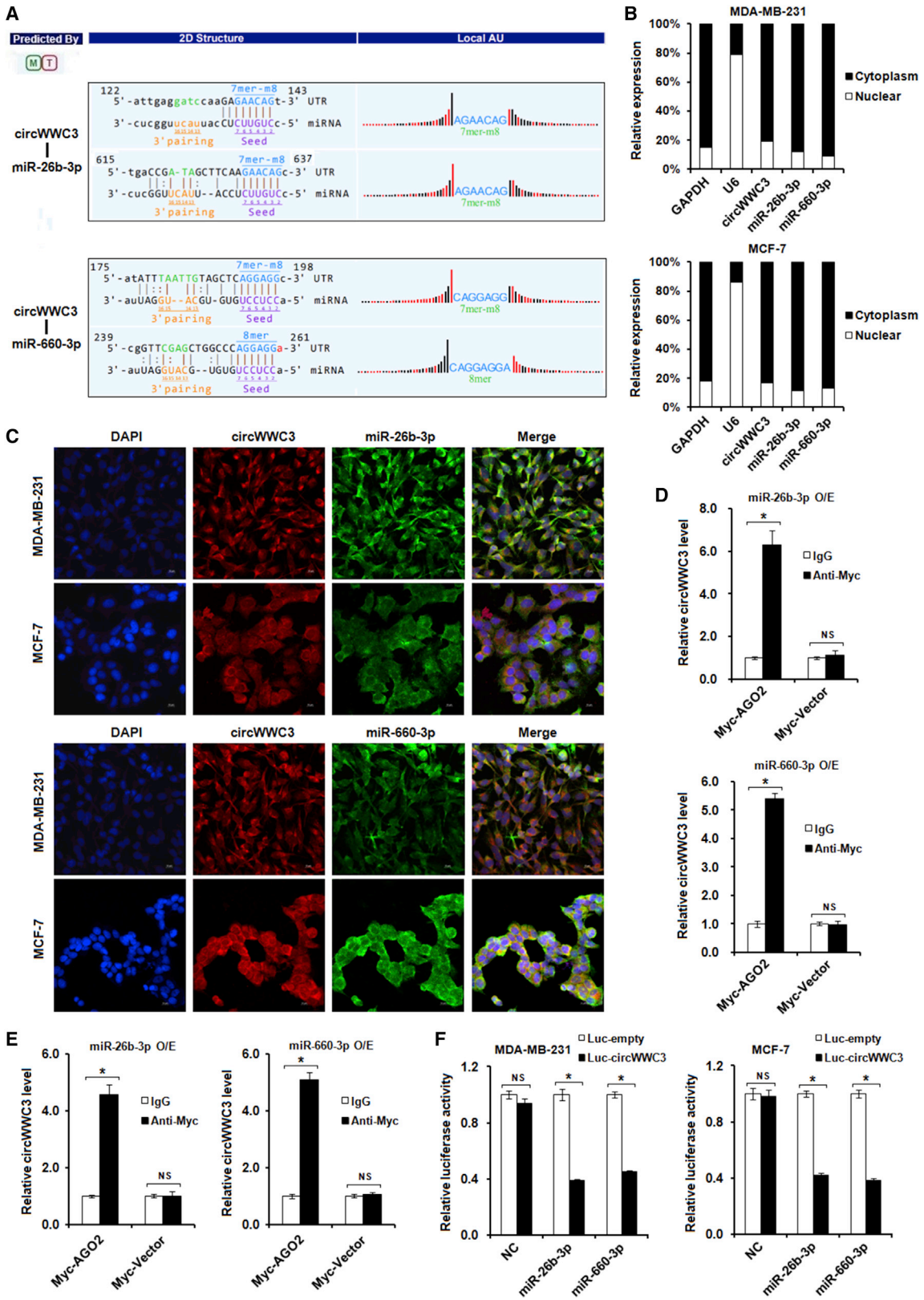
To investigate the circWWC3 expression in breast cancer tissues and its effect on breast cancer malignancy, we randomly selected 156 cases of breast cancer tissues and examined the expression of circWWC3 using FISH (Figure 2A). As shown in Table S4, circWWC3 expression is positively associated with the clinical stage of breast cancer patients, suggesting that upregulation of circWWC3 indicates an aggressive characteristic of breast cancer. In addition, the survival analysis showed that elevated expression of circWWC3 indicated a poor overall survival of breast cancer patients (Figure 2B). These results demonstrated that circWWC3 may play an oncogenic role in breast cancer progression.

circWWC3 Increases Cell Proliferation, Migration, and Invasion of Breast Cancer Cells

To investigate the biological role of circWWC3 in breast cancer progression, we used RNA interference to silence the expression of circWWC3 in two circWWC3 high-expressed breast cancer cell lines. We designed two small interfering (si)RNAs to target the back-splice sequence of circWWC3 that did not affect the expression of the WWC3 linear species (Figure 2C). Subsequent functional experiments revealed that downregulation of circWWC3 significantly suppressed proliferation, migration, and invasion of MDA-MB-231 and

Figure 2. circWWC3 Is Associated with Breast Cancer Progression and Increases Proliferation, Migration, and Invasion of Breast Cancer Cells

(A) The expression of circWWC3 in breast cancer tissues was detected by FISH analysis. (B) The survival analysis showed that elevated expression of circWWC3 indicated a poor overall survival of breast cancer patients. (C) The expression of circWWC3 and linear WWC3 in MDA-MB-231 and MCF-7 cells after transfection with siRNAs against circWWC3 was detected by qRT-PCR. *$p < 0.05$. (D and H) Cell Counting Kit 8 (CCK8) assay revealed that downregulation of circWWC3 significantly suppressed proliferation of MDA-MB-231 (D) and MCF-7 (H) cells. *$p < 0.05$. (E and I) Colony-formation assay showed that the colony-forming ability was significantly reduced after downregulation of circWWC3 expression in MDA-MB-231 (E) and MCF-7 (I) cells. *$p < 0.05$. (F and J) Wound-healing experiment showed that downregulation of circWWC3 inhibited migration of both MDA-MB-231 (F) and MCF-7 (J) cells. *$p < 0.05$. (G and K) Transwell migration and Matrigel invasion assay revealed that downregulation of circWWC3 inhibited migration and invasion of MDA-MB-231 (G) and MCF-7 (K) cells. *$p < 0.05$.



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MCF-7 cells (Figures 2D–2K). These evidences implied that circWWC3 may function as a facilitating factor for breast cancer progression.

We also used RNA interference to knock down the expression of linear WWC3 or circWWC3 plus linear WWC3 (Figure S3A) in MDA-MB-231 cells. Cell proliferation, migration, and invasion were not changed when both linear WWC3 and circWWC3 were knocked down. siRNA-mediated knockdown of linear WWC3 increased proliferation, migration, and invasion of breast cancer cells (Figures S3B–S3E). Survival analysis from the Kaplan-Meier Plotter database showed that downregulation of linear WWC3 expression was associated with the poor survival of breast cancer patients (Figure S3F). These data revealed that contrary to circWWC3, the linear form of WWC3 plays a tumor-suppressive role in breast cancer progression. In addition, overexpression of circWWC3 increased proliferation, migration, and invasion of MDA-MB-453 cells (Figures S4A–S4E). Taken together, our results demonstrated that circWWC3 increases proliferation, migration, and invasion of breast cancer cells and plays oncogenic functions in breast cancer progression.

circWWC3 Functions as the Sponge of miR-26b-3p and miR-660-3p

Because circWWC3 has a high abundance in breast cancer and is located in the cytoplasm, we explored whether circWWC3 binds to miRNAs as a miRNA sponge. Through analyzing the miRanda and TargetScan databases, the top 2 miRNAs were miR-26b-3p and miR-660-3p, based on the miRNA response element (MRE) analysis of putative binding sites in the circWWC3 sequence (Figure 3A). We first detected the colocalization of circWWC3, miR-26b-3p, and miR-660-3p in MDA-MB-231 and MCF-7 cells. qRT-PCR and FISH results revealed that both circWWC3 and miR-26b-3p or miR-660-3p were predominantly located in the cytoplasm (Figures 3B and 3C), which suggested that circWWC3 has a condition to act as the sponge of miR-26b-3p and miR-660-3p. RNA-binding protein immunoprecipitation (RIP) results revealed that circWWC3 could interact with miR-26b-3p and miR-660-3p in breast cancer MDA-MB-231 and MCF-7 cells (Figures 3D and 3E). The luciferase reporter assay showed that both miR-26b-3p and miR-660-3p inhibited the luciferase activity of luciferase-circWWC3 in both MDA-MB-231 and MCF-7 cells (Figure 3F). These data suggested that circWWC3 may serve as a sponge for both miR-26b-3p and miR-660-3p.

circWWC3 Maintained the Oncogenic Properties of Breast Cancer Cells through Acting as the Sponge of miR-26b-3p and miR-660-3p

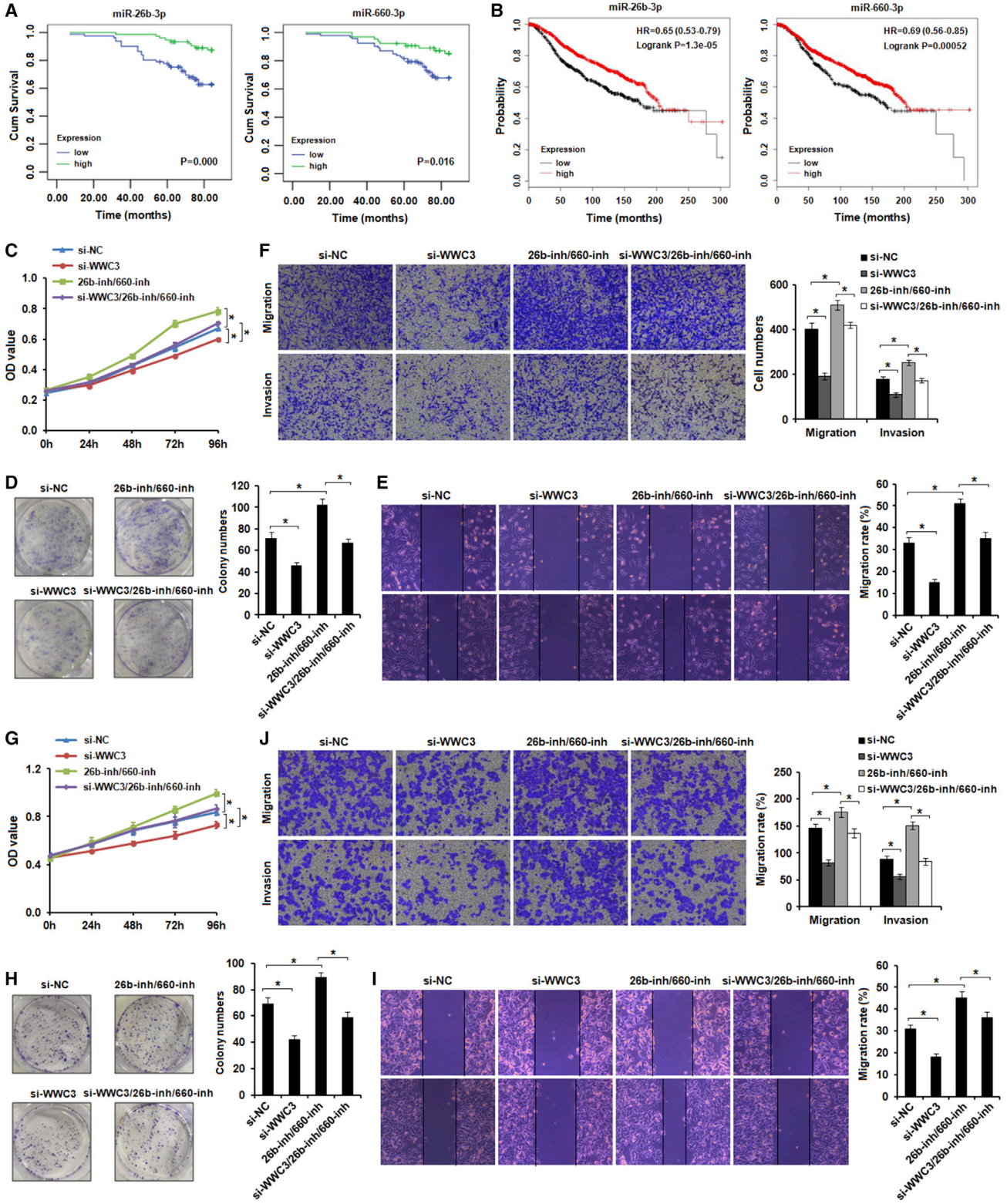
To investigate the possible role of miR-26b-3p and miR-660-3p in breast cancer, we examined their expression using qRT-PCR and analyzed the effect of miR-26b-3p or miR-660-3p on the overall survival of breast cancer patients. Our results revealed that lower expression of miR-26b-3p and miR-660-3p indicated poor overall survival of breast cancer patients (Figure 4A). Our results were supported by the Kaplan-Meier Plotter database (Figure 4B). Functional experiments revealed that enforced expression of miR-26b-3p or miR-660-3p inhibited cell proliferation, migration, and invasion of MDA-MB-453 cells (Figures S5A–S5F). In miR-26b-3p or miR-660-3p high-expressed MDA-MB-231 and BT-549 cells, miR-26b-3p or miR-660-3p inhibitors increased cell proliferation, migration, and invasion (Figures S6A–S6H). Our results suggested that miR-26b-3p and miR-660-3p might play a tumor-suppressive role in breast cancer progression. The rescue experiment in MDA-MB-231 cells revealed that circWWC3 suppression inhibited cell proliferation, migration, and invasion, whereas miR-26b-3p and miR-660-3p suppression rescued circWWC3 knockdown-mediated inhibition of cell proliferation, migration, and invasion (Figures 4C–4F). Similar results were obtained in MCF-7 cells (Figures 4G–4J). These results suggested that high expression of circWWC3 probably maintained the oncogenic properties of breast cancer cells through sponging miR-26b-3p and miR-660-3p. siRNA-mediated knockdown of circWWC3 suppressed the oncogenic properties through releasing miR-26b-3p and miR-660-3p. miR-26b-3p and miR-660-3p inhibitors antagonized the circWWC3 siRNA-mediated release of these two miRNAs. Taken together, our results indicated that circWWC3 probably maintained the oncogenic properties of breast cancer cells through antagonizing the tumor-suppressive role of miR-26b-3p and miR-660-3p in breast cancer.

Ras Signaling Pathway Is the Direct Pathway of miR-26b-3p and miR-660-3p in Breast Cancer Cells and Is Inhibited via circWWC3 Knockdown

To gain further insight into the downstream pathways regulated by miR-26b-3p and miR-660-3p, we identified 2,234 putative cotarget genes of miR-26b-3p and miR-660-3p through the TargetScan database (Figure 5A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that 45 pathways were associated with the cotarget genes of miR-26b-3p and miR-660-3p (Figure S7). In these pathways, "Ras signaling pathway" was most significantly associated with these target genes. Most of these genes are shown to have oncogenic potential in cancer progression, such as EGFR, GRB2, PAK4, MAPK1, and AKT1. Through the TargetScan database, multiple potential binding sites for miR-26b-3p and miR-660-3p seed regions on the 3′ UTR of these target genes were found (Figure S8). qRT-PCR and western blot results revealed that miR-26b-3p and miR-660-3p did not change the mRNA level (Figure 5B) but notably inhibited the protein expression of these genes in both MDA-MB-231 and MCF-7 cells (Figures 5C and 5D). Furthermore, we found that

Figure 3. circWWC3 Functions as the Sponge of miR-26b-3p and miR-660-3p

(A) The putative binding sites of miR-26b-3p and miR-660-3p in circWWC3 was analyzed by miRanda and TargetScan databases. (B and C) qRT-PCR (B) and FISH (C) results revealed that both circWWC3 and miR-26b-3p or miR-660-3p were predominantly located in cytoplasm. (D and E) RNA-binding protein immunoprecipitation (RIP) results revealed that circWWC3 could interact with miR-26b-3p and miR-660-3p in MDA-MB-231 (D) and MCF-7 (E) cells. *$p < 0.05$; NS, $p > 0.05$. (F) Dual-luciferase reporter assay showed that both miR-26b-3p and miR-660-3p inhibited the luciferase activity of luciferase (Luc)-circWWC3 in both MDA-MB-231 and MCF-7 cells. *$p < 0.05$; NS, $p > 0.05$.



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knockdown of circWWC3 also reduced the protein expression of these genes (Figures 5E and 5F). FISH and immunohistochemistry (IHC) results revealed that high expression of circWWC3 was associated with the high expression of these target genes (Figure 6A; Table S5). Kaplan-Meier analysis showed high expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 was associated with the poor overall survival of breast cancer patients (Figure 6B), which was supported by Kaplan-Meier Plotter database results (Figure 6C). Taken together, our results indicated that the Ras signaling pathway is the direct pathway of miR-26b-3p and miR-660-3p in breast cancer cells and is inhibited through circWWC3 knockdown.

ZEB1 Promotes Breast Cancer Growth and Metastasis through Regulating the circWWC3-miR-26b-3p/miR-660-3p-Ras Signaling Pathway Axis *In Vivo*

To investigate whether the ZEB1-circWWC3-miR-26b-3p/miR-660-3p-Ras signaling pathway axis affects breast cancer growth and metastasis, we applied the preclinical study using the *in vivo* mouse breast cancer xenograft and metastasis model with MDA-MB-231 cells expressing firefly luciferase. Female nude mice were randomly divided into four groups, as indicated in Materials and Methods. For the xenograft tumor model, an increase of the tumor luciferase signal in the ZEB1-infected group was found after 3 weeks (Figure 7A). According to the results, enforced expression of ZEB1 increased the tumor growth of breast cancer and enhanced the expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 (Figures 7B–7D). shRNA-mediated knockdown of circWWC3 could partly reverse the ZEB1-induced breast cancer growth and the expression of the above proteins (Figures 7B–7D). For the metastasis model, mice were sacrificed after 8 weeks, and the metastatic foci in lung and liver were observed by IVIS and counted by the naked eye (Figures 7E and 7F). The metastasis was further confirmed by H&E staining (Figure 7G). The results revealed that overexpression of ZEB1 increased the metastasis of breast cancer, whereas shRNA-mediated knockdown of circWWC3 partly reversed the ZEB1-induced breast cancer metastasis. Furthermore, ZEB1 expression was positively correlated with the expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 (Table S6). The survival analysis revealed that high expression of ZEB1 plus circWWC3 indicated a poor prognosis of breast cancer patients (Figure 7H). Taken together, these results proved that ZEB1 may play a promoting role on breast cancer growth and metastasis via modulation of the circWWC3-miR-26b-3p/miR-660-3p-Ras signaling pathway axis.

DISCUSSION

Accumulating evidence demonstrated that circRNAs play crucial roles in the development of breast cancer.[21–25] circRNAs have been proven to be generated cotranscriptionally and compete with the canonical pre-mRNA splicing.[16,26] As a zinc-finger transcription factor, ZEB1 has been widely recognized as an important driver of tumor growth and metastasis in cancer, especially in breast cancer.[27] However, the role of ZEB1-regulated circRNAs in breast cancer progression still remains unexplored. In the present study, we examined the ZEB1-regulated transcription profile of circRNAs. As a result, we found that exogenous expression of ZEB1 induced the transcription of the circular form of WWC3 but not the linear WWC3. circWWC3 is highly abundant in breast cancer and was associated with a poor prognosis of breast cancer patients. Mechanically, circWWC3 promoted the tumor growth and metastasis of breast cancer through acting as the sponge of miR-26b-3p and miR-660-3p to target the Ras signaling pathway. Our results presented evidence that activation of ZEB1 and ZEB1-induced circWWC3 is important for breast cancer progression.

WWC3 has been identified as a tumor suppressor that is downregulated in several malignancies. Downregulation of WWC3 is associated with poor prognosis of cancer patients.[28–30] WWC3 inhibits cell proliferation and metastasis through regulating the Hippo and Wnt signaling pathways.[28–31] In our present study, linear WWC3 was associated with good prognosis and inhibited breast cancer cell growth and metastasis. However, the circular form of WWC3 was dominant and exhibited oncogenic functions in breast cancer, indicating that the competition of circWWC3 with the linear WWC3 promoted the progression of breast cancer. Further studies are required to elucidate the mechanism of backsplicing formation of WWC3 pre-mRNA in breast cancer.

Due to the high abundance, stability, and potential number of MREs that they contain, some circRNAs could function as "miRNA sponges."[8,11,32–38] Our study revealed that circWWC3 was abundant in breast cancer and promoted the breast cancer growth and metastasis through acting as the sponge of miR-26b-3p and miR-660-3p to target the Ras signaling pathway. These findings revealed that circRNAs could act as sponges of miRNAs and thereby regulate cancer progression. Of note, not all circRNAs function as miRNA sponges in cells. At present, several functional models of circRNAs have been reported: (1) circRNAs function as miRNA sponges, which competitively bind endogenous miRNAs; (2) circRNAs function as "protein scaffolds," which means that circRNAs directly bind to proteins and maintain the stability of the protein complex;[39–42] and (3) some circRNAs can be translated as "coding RNAs," which means that they directly encode a protein or peptide.[43–52] Due to the lack of free ends, circRNAs are resistant to exonucleases and more stable

Figure 4. circWWC3 Maintained the Oncogenic Properties of Breast Cancer Cells through Acting as the Sponge of miR-26b-3p and miR-660-3p

(A) The lower expression of miR-26b-3p and miR-660-3p indicated poor overall survival of breast cancer patients in our study. (B) The Kaplan-Meier Plotter database (http://kmplot.com/analysis/) was used to validate our survival results. (C–F) circWWC3 suppression inhibited cell proliferation (C and D) and migration and invasion (E and F), whereas miR-26b-3p and miR-660-3p suppression rescued circWWC3 knockdown-mediated inhibition of proliferation, migration, and invasion of MDA-MB-231 cells. (G–J) circWWC3 suppression inhibited cell proliferation (G and H) and migration and invasion (I and J), whereas miR-26b-3p and miR-660-3p suppression rescued circWWC3 knockdown-mediated inhibition of proliferation, migration, and invasion of MCF-7 cells. *$p < 0.05$.

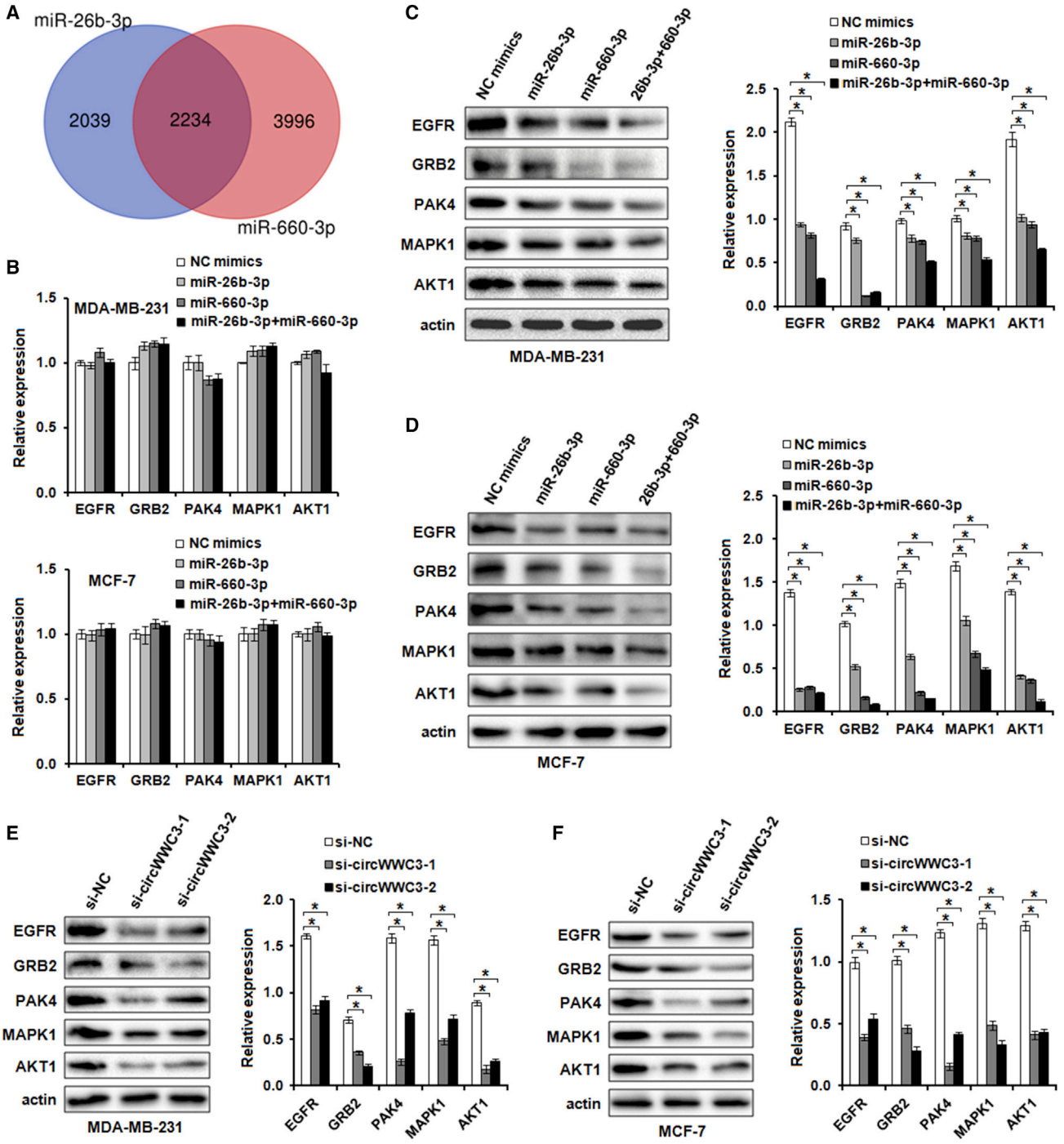


Figure 5. Ras Signaling Pathway Is the Direct Pathway of miR-26b-3p and miR-660-3p in Breast Cancer Cells and Is Inhibited via circWWC3 Knockdown

(A) 2,234 putative cotarget genes of miR-26b-3p and miR-660-3p were identified through the TargetScan database. (B) qRT-PCR was performed to detect the mRNA expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 after transfected miR-26b-3p and miR-660-3p mimics into MDA-MB-231 or MCF-7 cells. (C and D) Western blot was performed to detect the protein expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 after transfected miR-26b-3p and miR-660-3p mimics into MDA-MB-231 (C) or MCF-7 (D) cells. *$p < 0.05$. (E and F) Knockdown of circWWC3 reduced the protein expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 in MDA-MB-231 (E) and MCF-7 (F) cells. *$p < 0.05$.

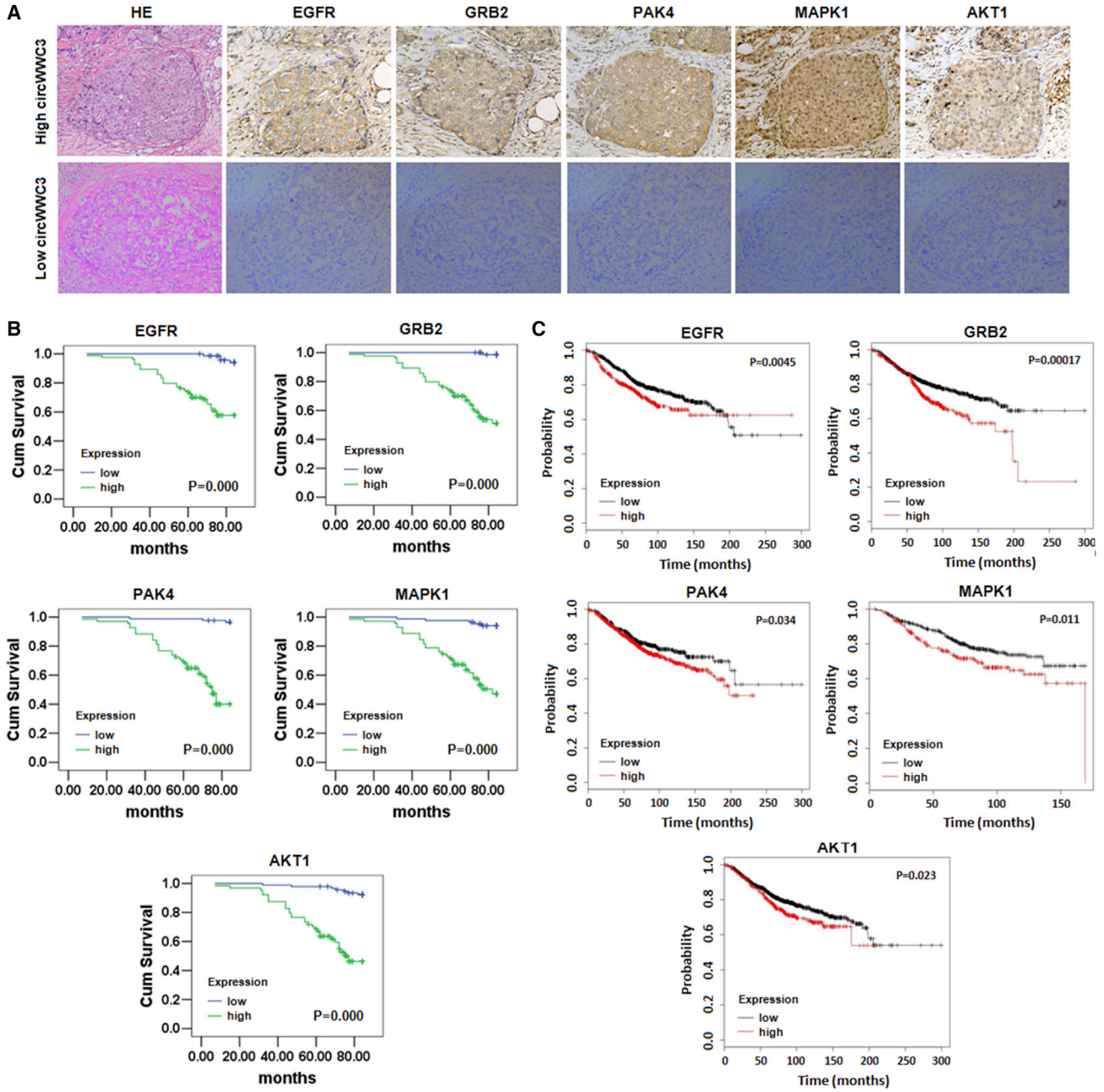
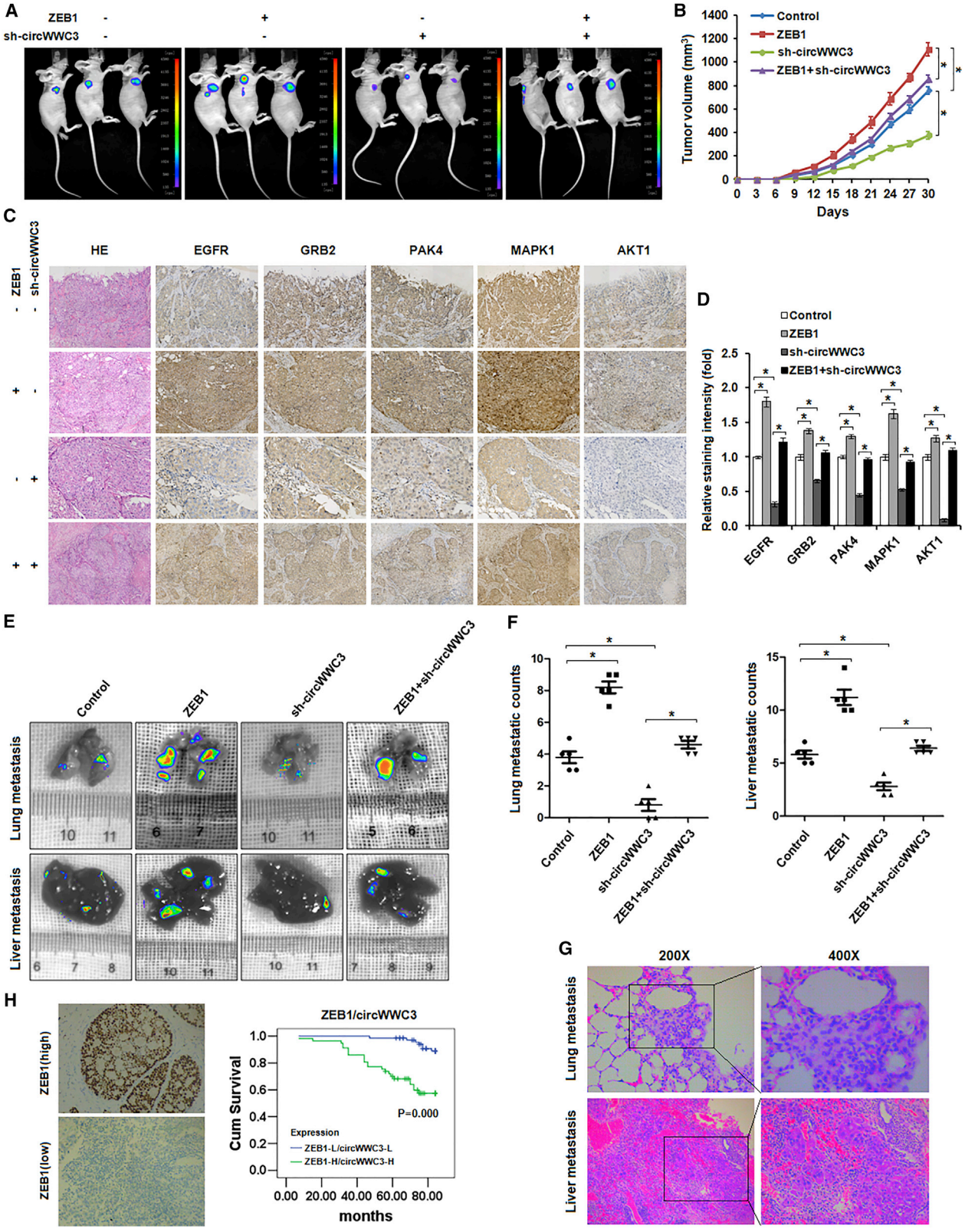


Figure 6. High Expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 Was Associated with the Poor Overall Survival of Breast Cancer Patients

(A) Immunohistochemistry (IHC) was performed to detect the expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 in the breast cancer tissues with high or low expression of circWWC3. (B) High expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 was associated with the poor overall survival of breast cancer patients in our study. (C) Kaplan-Meier Plotter database (http://kmplot.com/analysis/) was used to validate our results.

than their linear isoforms. Moreover, some small circRNAs can be absorbed into exosomes. Therefore, circRNAs have the potential to function as the predicting markers for cancer diagnosis and prognosis.[53–56] Therefore, the functions of circRNAs in cancer progression still need to be further explored.

In summary, our study can be concluded by the following major findings: (1) We identified a novel circRNA, circWWC3, that is induced by ZEB1 and promotes breast cancer growth and metastasis. (2) circWWC3 can act as the sponge of miR-26b-3p and miR-660-3p and inhibit their tumor-suppressive functions in breast cancer. (3)



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The Ras signaling pathway is the target pathway of the circWWC3-miR-26b-3p/miR-660-3p axis in breast cancer. (4) ZEB1-mediated upregulation of circWWC3 promotes breast cancer progression through activating the Ras signaling pathway. Our study revealed that circWWC3 may function as an oncogene and act as a prognostic biomarker and therapeutic target for breast cancer.

MATERIALS AND METHODS

circRNA Microarray

Total RNA was extracted from MDA-MB-231 cells transfected with ZEB1 or empty vector. Arraystar Human Circular RNA Microarray v.2 (catalog number: AS-CR-H-V2.0; Arraystar, MD, USA) was used to identify differentially expressed circRNAs after transfection of ZEB1 in MDA-MB-231 cells. The sample preparation and microarray hybridization process were carried out based on Arraystar's protocols.

Clinical Specimens

All human breast cancer tissues were collected from the Fourth Hospital of Hebei Medical University. All patients did not receive preoperative chemotherapy and radiation therapy. The human tissues were obtained with informed consent, and our study was approved by the Clinical Research Ethics Committee of our hospital.

Animal Experiment

The animal experiments were approved by Animal Care Committee of the Fourth Hospital of Hebei Medical University. For xenograft tumor experiment and *in vivo* metastasis experiment, 4-week-old female BALB/c nude mice were randomly divided into four groups (n = 5 for each group). MDA-MB-231-luciferase cells (1×10^6 cells per mouse) stably transfected with (1) empty vector + sh-scramble, (2) ZEB1 + sh-scramble, (3) empty vector + sh-circWWC3, or (4) ZEB1 + sh-circWWC3 were injected into mice subcutaneously for xenograft model or via tail vein and spleen for lung and liver metastasis model establishment. Tumors were evaluated weekly by using an *in vivo* imaging system (IVIS). Two months later, mice were sacrificed. The lung and liver were observed by the naked eye and H&E staining.

Statistical Analysis

All statistical analyses were performed with SPSS 22.0 (SPSS, Chicago, IL, USA). The quantitative data were analyzed by Student's t test. The clinicopathological analysis was performed by chi-square test. The overall survival was evaluated by the Kaplan-Meier method. All statistical analyses were two sided. p value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.08.015.

AUTHOR CONTRIBUTIONS

Study Concept and Design, L.M., C.G., and M.S.; Specimen Provider, X.F., L.G., and M.S.; Acquisition of Clinical Data, F.L., S.L., and Z.L.; Analysis and Interpretation of Data and Statistical Analysis, L.M., S.L., and M.S.; Animal Experiments, L.M. and Y.J.; Drafting of the Manuscript, L.M. and M.S.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. Hugo, H.J., Pereira, L., Suryadinata, R., Drabsch, Y., Gonda, T.J., Gunasinghe, N.P., Pinto, C., Soo, E.T., van Denderen, B.J., Hill, P., et al. (2013). Direct repression of MYB by ZEB1 suppresses proliferation and epithelial gene expression during epithelial-to-mesenchymal transition of breast cancer cells. Breast Cancer Res. *15*, R113.
2. Chaffer, C.L., Marjanovic, N.D., Lee, T., Bell, G., Kleer, C.G., Reinhardt, F., D'Alessio, A.C., Young, R.A., and Weinberg, R.A. (2013). Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. Cell *154*, 61–74.
3. Liu, L., Tong, Q., Liu, S., Cui, J., Zhang, Q., Sun, W., and Yang, S. (2016). ZEB1 Upregulates VEGF Expression and Stimulates Angiogenesis in Breast Cancer. PLoS ONE *11*, e0148774.
4. Zhang, X., Zhang, Z., Zhang, Q., Zhang, Q., Sun, P., Xiang, R., Ren, G., and Yang, S. (2018). ZEB1 confers chemotherapeutic resistance to breast cancer by activating ATM. Cell Death Dis. *9*, 57.
5. Zhou, C., Jiang, H., Zhang, Z., Zhang, G., Wang, H., Zhang, Q., Sun, P., Xiang, R., and Yang, S. (2017). ZEB1 confers stem cell-like properties in breast cancer by targeting neurogenin-3. Oncotarget *8*, 54388–54401.
6. Agajanian, M., Runa, F., and Kelber, J.A. (2015). Identification of a PEAK1/ZEB1 signaling axis during TGFβ/fibronectin-induced EMT in breast cancer. Biochem. Biophys. Res. Commun. *465*, 606–612.
7. Karihtala, P., Auvinen, P., Kauppila, S., Haapasaari, K.M., Jukkola-Vuorinen, A., and Soini, Y. (2013). Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: association with an aggressive tumour phenotype. Breast Cancer Res. Treat. *138*, 81–90.
8. Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S.D., Gregersen, L.H., Munschauer, M., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature *495*, 333–338.
9. Jeck, W.R., and Sharpless, N.E. (2014). Detecting and characterizing circular RNAs. Nat. Biotechnol. *32*, 453–461.

Figure 7. ZEB1 Promotes Breast Cancer Growth and Metastasis through Regulating the circWWC3-miR-26b-3p/miR-660-3p-Ras Signaling Pathway Axis *In Vivo*

(A) An *in vivo* imaging system (IVIS) was performed to check the fluorescence signal of xenograft tumors. The representative bioluminescent images of three nude mice in each group were shown. (B) Tumor growth curve within 30 days was shown. *p < 0.05. (C and D) The expression of EGFR, GRB2, PAK4, MAPK1, and AKT1 in mice tumor tissues was examined by IHC. (C): Representative images; (D): Quantitative analysis. (E and F) The metastatic foci in lung and liver were observed by IVIS and counted by the naked eye. *p < 0.05. (E): Representative images; (F): Quantitative analysis. (G) The metastasis was further confirmed by H&E staining. (H) The survival analysis revealed that high expression of ZEB1 plus circWWC3 indicated a poor prognosis survival of breast cancer patients.

10. Lasda, E., and Parker, R. (2014). Circular RNAs: diversity of form and function. RNA *20*, 1829–1842.

11. Hansen, T.B., Jensen, T.I., Clausen, B.H., Bramsen, J.B., Finsen, B., Damgaard, C.K., and Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature *495*, 384–388.

12. Fang, L., Du, W.W., Awan, F.M., Dong, J., and Yang, B.B. (2019). The circular RNA circ-Ccnb1 dissociates Ccnb1/Cdk1 complex suppressing cell invasion and tumorigenesis. Cancer Lett. *459*, 216–226.

13. Chen, L., Nan, A., Zhang, N., Jia, Y., Li, X., Ling, Y., Dai, J., Zhang, S., Yang, Q., Yi, Y., and Jiang, Y. (2019). Circular RNA 100146 functions as an oncogene through direct binding to miR-361-3p and miR-615-5p in non-small cell lung cancer. Mol. Cancer *18*, 13.

14. Barbagallo, D., Caponnetto, A., Brex, D., Mirabella, F., Barbagallo, C., Lauretta, G., Morrone, A., Certo, F., Broggi, G., Caltabiano, R., et al. (2019). CircSMARCA5 Regulates VEGFA mRNA Splicing and Angiogenesis in Glioblastoma Multiforme Through the Binding of SRSF1. Cancers (Basel) *11*, E194.

15. Barbagallo, D., Caponnetto, A., Cirnigliaro, M., Brex, D., Barbagallo, C., D'Angeli, F., Morrone, A., Caltabiano, R., Barbagallo, G.M., Ragusa, M., et al. (2018). CircSMARCA5 Inhibits Migration of Glioblastoma Multiforme Cells by Regulating a Molecular Axis Involving Splicing Factors SRSF1/SRSF3/PTB. Int. J. Mol. Sci. *19*, E480.

16. Ashwal-Fluss, R., Meyer, M., Pamudurti, N.R., Ivanov, A., Bartok, O., Hanan, M., Evantal, N., Memczak, S., Rajewsky, N., and Kadener, S. (2014). circRNA biogenesis competes with pre-mRNA splicing. Mol. Cell *56*, 55–66.

17. Han, Z., Zhang, Y., Sun, Y., Chen, J., Chang, C., Wang, X., and Yeh, S. (2018). ERβ-Mediated Alteration of circATP2B1 and miR-204-3p Signaling Promotes Invasion of Clear Cell Renal Cell Carcinoma. Cancer Res. *78*, 2550–2563.

18. Wang, K., Sun, Y., Tao, W., Fei, X., and Chang, C. (2017). Androgen receptor (AR) promotes clear cell renal cell carcinoma (ccRCC) migration and invasion via altering the circHIAT1/miR-195-5p/29a-3p/29c-3p/CDC42 signals. Cancer Lett. *394*, 1–12.

19. Meng, J., Chen, S., Han, J.X., Qian, B., Wang, X.R., Zhong, W.L., Qin, Y., Zhang, H., Gao, W.F., Lei, Y.Y., et al. (2018). Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma. Cancer Res. *78*, 4150–4162.

20. Ruan, H., Xiang, Y., Ko, J., Li, S., Jing, Y., Zhu, X., Ye, Y., Zhang, Z., Mills, T., Feng, J., et al. (2019). Comprehensive characterization of circular RNAs in ∼ 1000 human cancer cell lines. Genome Med. *11*, 55.

21. Chen, N., Zhao, G., Yan, X., Lv, Z., Yin, H., Zhang, S., Song, W., Li, X., Li, L., Du, Z., et al. (2018). A novel FLI1 exonic circular RNA promotes metastasis in breast cancer by coordinately regulating TET1 and DNMT1. Genome Biol. *19*, 218.

22. Chen, B., Wei, W., Huang, X., Xie, X., Kong, Y., Dai, D., Yang, L., Wang, J., Tang, H., and Xie, X. (2018). circEPSTI1 as a prognostic marker and mediator of triple-negative breast cancer progression. Theranostics *8*, 4003–4015.

23. Sang, Y., Chen, B., Song, X., Li, Y., Liang, Y., Han, D., Zhang, N., Zhang, H., Liu, Y., Chen, T., et al. (2019). circRNA_0025202 Regulates Tamoxifen Sensitivity and Tumor Progression via Regulating the miR-182-5p/FOXO3a Axis in Breast Cancer. Mol. Ther. *27*, 1638–1652.

24. Fang, L., Du, W.W., Lyu, J., Dong, J., Zhang, C., Yang, W., He, A., Kwok, Y.S.S., Ma, J., Wu, N., et al. (2018). Enhanced breast cancer progression by mutant p53 is inhibited by the circular RNA circ-Ccnb1. Cell Death Differ. *25*, 2195–2208.

25. Tang, H., Huang, X., Wang, J., Yang, L., Kong, Y., Gao, G., Zhang, L., Chen, Z.S., and Xie, X. (2019). circKIF4A acts as a prognostic factor and mediator to regulate the progression of triple-negative breast cancer. Mol. Cancer *18*, 23.

26. Sibley, C.R., Blazquez, L., and Ule, J. (2016). Lessons from non-canonical splicing. Nat. Rev. Genet. *17*, 407–421.

27. Fu, R., Han, C.F., Ni, T., Di, L., Liu, L.J., Lv, W.C., Bi, Y.R., Jiang, N., He, Y., Li, H.M., et al. (2019). A ZEB1/p53 signaling axis in stromal fibroblasts promotes mammary epithelial tumours. Nat. Commun. *10*, 3210.

28. Han, Q., Lin, X., Zhang, X., Jiang, G., Zhang, Y., Miao, Y., Rong, X., Zheng, X., Han, Y., Han, X., et al. (2017). WWC3 regulates the Wnt and Hippo pathways via Dishevelled proteins and large tumour suppressor 1, to suppress lung cancer invasion and metastasis. J. Pathol. *242*, 435–447.

29. Wang, Y., Jiang, M., Yao, Y., and Cai, Z. (2018). WWC3 Inhibits Glioma Cell Proliferation Through Suppressing the Wnt/β-Catenin Signaling Pathway. DNA Cell Biol. *37*, 31–37.

30. Hou, J., and Zhou, J. (2017). WWC3 downregulation correlates with poor prognosis and inhibition of Hippo signaling in human gastric cancer. OncoTargets Ther. *10*, 2931–2942.

31. Han, Q., Kremerskothen, J., Lin, X., Zhang, X., Rong, X., Zhang, D., and Wang, E. (2018). WWC3 inhibits epithelial-mesenchymal transition of lung cancer by activating Hippo-YAP signaling. OncoTargets Ther. *11*, 2581–2591.

32. Tay, Y., Rinn, J., and Pandolfi, P.P. (2014). The multilayered complexity of ceRNA crosstalk and competition. Nature *505*, 344–352.

33. Piwecka, M., Glažar, P., Hernandez-Miranda, L.R., Memczak, S., Wolf, S.A., Rybak-Wolf, A., Filipchyk, A., Klironomos, F., Cerda Jara, C.A., Fenske, P., et al. (2017). Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. Science *357*, eaam8526.

34. Sang, M., Meng, L., Sang, Y., Liu, S., Ding, P., Ju, Y., Liu, F., Gu, L., Lian, Y., Li, J., et al. (2018). Circular RNA ciRS-7 accelerates ESCC progression through acting as a miR-876-5p sponge to enhance MAGE-A family expression. Cancer Lett. *426*, 37–46.

35. Sang, M., Meng, L., Liu, S., Ding, P., Chang, S., Ju, Y., Liu, F., Gu, L., Lian, Y., and Geng, C. (2018). Circular RNA ciRS-7 Maintains Metastatic Phenotypes as a ceRNA of miR-1299 to Target MMPs. Mol. Cancer Res. *16*, 1665–1675.

36. Meng, L., Liu, S., Ding, P., Chang, S., and Sang, M. (2020). Circular RNA ciRS-7 inhibits autophagy of ESCC cells by functioning as miR-1299 sponge to target EGFR signaling. J. Cell. Biochem. *121*, 1039–1049.

37. Zheng, Q., Bao, C., Guo, W., Li, S., Chen, J., Chen, B., Luo, Y., Lyu, D., Li, Y., Shi, G., et al. (2016). Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. Nat. Commun. *7*, 11215.

38. Han, D., Li, J., Wang, H., Su, X., Hou, J., Gu, Y., Qian, C., Lin, Y., Liu, X., Huang, M., et al. (2017). Circular RNA circMTO1 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology *66*, 1151–1164.

39. Du, W.W., Yang, W., Liu, E., Yang, Z., Dhaliwal, P., and Yang, B.B. (2016). Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. Nucleic Acids Res. *44*, 2846–2858.

40. Wang, L., Long, H., Zheng, Q., Bo, X., Xiao, X., and Li, B. (2019). Circular RNA circRHOT1 promotes hepatocellular carcinoma progression by initiation of NR2F6 expression. Mol. Cancer *18*, 119.

41. Kong, P., Yu, Y., Wang, L., Dou, Y.Q., Zhang, X.H., Cui, Y., Wang, H.Y., Yong, Y.T., Liu, Y.B., Hu, H.J., et al. (2019). circ-Sirt1 controls NF-κB activation via sequence-specific interaction and enhancement of SIRT1 expression by binding to miR-132/212 in vascular smooth muscle cells. Nucleic Acids Res. *47*, 3580–3593.

42. Yang, F., Fang, E., Mei, H., Chen, Y., Li, H., Li, D., Song, H., Wang, J., Hong, M., Xiao, W., et al. (2019). *Cis*-Acting *circ-CTNNB1* Promotes β-Catenin Signaling and Cancer Progression via DDX3-Mediated Transactivation of YY1. Cancer Res. *79*, 557–571.

43. Pamudurti, N.R., Bartok, O., Jens, M., Ashwal-Fluss, R., Stottmeister, C., Ruhe, L., Hanan, M., Wyler, E., Perez-Hernandez, D., Ramberger, E., et al. (2017). Translation of CircRNAs. Mol. Cell *66*, 9–21.e7.

44. Legnini, I., Di Timoteo, G., Rossi, F., Morlando, M., Briganti, F., Sthandier, O., Fatica, A., Santini, T., Andronache, A., Wade, M., et al. (2017). Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis. Mol. Cell *66*, 22–37.e9.

45. Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., Jin, Y., Yang, Y., Chen, L.L., Wang, Y., et al. (2017). Extensive translation of circular RNAs driven by N^6-methyladenosine. Cell Res. *27*, 626–641.

46. Zheng, X., Chen, L., Zhou, Y., Wang, Q., Zheng, Z., Xu, B., Wu, C., Zhou, Q., Hu, W., Wu, C., and Jiang, J. (2019). A novel protein encoded by a circular RNA circPPP1R12A promotes tumor pathogenesis and metastasis of colon cancer via Hippo-YAP signaling. Mol. Cancer *18*, 47.

47. Xia, X., Li, X., Li, F., Wu, X., Zhang, M., Zhou, H., Huang, N., Yang, X., Xiao, F., Liu, D., et al. (2019). A novel tumor suppressor protein encoded by circular AKT3 RNA inhibits glioblastoma tumorigenicity by competing with active phosphoinositide-dependent Kinase-1. Mol. Cancer *18*, 131.

48. Zhang, M., Zhao, K., Xu, X., Yang, Y., Yan, S., Wei, P., Liu, H., Xu, J., Xiao, F., Zhou, H., et al. (2018). A peptide encoded by circular form of LINC-PINT suppresses oncogenic transcriptional elongation in glioblastoma. Nat. Commun. *9*, 4475.

49. Yang, Y., Gao, X., Zhang, M., Yan, S., Sun, C., Xiao, F., Huang, N., Yang, X., Zhao, K., Zhou, H., et al. (2018). Novel Role of FBXW7 Circular RNA in Repressing Glioma Tumorigenesis. J. Natl. Cancer Inst. *110*, 304–315.

50. Begum, S., Yiu, A., Stebbing, J., and Castellano, L. (2018). Novel tumour suppressive protein encoded by circular RNA, circ-SHPRH, in glioblastomas. Oncogene *37*, 4055–4057.

51. Zhang, M., Huang, N., Yang, X., Luo, J., Yan, S., Xiao, F., Chen, W., Gao, X., Zhao, K., Zhou, H., et al. (2018). A novel protein encoded by the circular form of the SHPRH gene suppresses glioma tumorigenesis. Oncogene *37*, 1805–1814.

52. Liang, W.C., Wong, C.W., Liang, P.P., Shi, M., Cao, Y., Rao, S.T., Tsui, S.K., Waye, M.M., Zhang, Q., Fu, W.M., and Zhang, J.F. (2019). Translation of the circular RNA circβ-catenin promotes liver cancer cell growth through activation of the Wnt pathway. Genome Biol. *20*, 84.

53. Li, Y., Zheng, Q., Bao, C., Li, S., Guo, W., Zhao, J., Chen, D., Gu, J., He, X., and Huang, S. (2015). Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell Res. *25*, 981–984.

54. Wang, F., Nazarali, A.J., and Ji, S. (2016). Circular RNAs as potential biomarkers for cancer diagnosis and therapy. Am. J. Cancer Res. *6*, 1167–1176.

55. Chen, J., Li, Y., Zheng, Q., Bao, C., He, J., Chen, B., Lyu, D., Zheng, B., Xu, Y., Long, Z., et al. (2017). Circular RNA profile identifies circPVT1 as a proliferative factor and prognostic marker in gastric cancer. Cancer Lett. *388*, 208–219.

56. Tan, S., Gou, Q., Pu, W., Guo, C., Yang, Y., Wu, K., Liu, Y., Liu, L., Wei, Y.Q., and Peng, Y. (2018). Circular RNA F-circEA produced from EML4-ALK fusion gene as a novel liquid biopsy biomarker for non-small cell lung cancer. Cell Res. *28*, 693–695.